Suicide Inactivation of Monoamine Oxidase by trans-Phenylcyclopropylamine*

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The mechanism of the inactivation of monoamine oxidase by arylcyclopropylamines has been studied using [2-14C]jpm-trans-phenylcyclopropylamine. This compound is a typical suicide inhibitor of bovine liver monoamine oxidase. It reacts rapidly and irreversibly with the enzyme, resulting in the incorporation of slightly over 1 mol of the inhibitor; the reaction is prevented but not reversed by substrates. Further, the enzyme oxidizes trans-phenylcyclopropylamine to the imine which, either as such or after hydrolysis to phenylcyclopropanone, combines irreversibly with the protein. Although inactivation is accompanied by bleaching of the 450 nm band of the covalently bound flavin component, which remains bleached even in the presence of air, the flavin ring system is not the site of combination of the inhibitor. This is shown by several lines of evidence. First, acid or heat denaturation releases all the 14C-labeled inhibitor and results in reoxidation of the flavin under conditions where adducts with N(5) or C(4a) of the flavin would be stable. Second, labeled inhibitor. It is suggested that the imine (or combination of the inhibitor. This is shown by several leases all the 14C-labeled inhibitor and results in reoxi-

dation of the flavin under conditions where adducts


Recognition of the cardinal role of monoamine oxidase in the catabolism of biogenic amines and of the usefulness of certain inhibitors of the enzyme in the treatment of mental depression has led to the development of a series of highly specific, irreversible inhibitors of the enzyme. The three main classes of active site-directed, irreversible inhibitors of mitochondrial monoamine oxidase are acetylenic amines, substituted hydrazines, and arylcyclopropylamines. Acetylenic amines and hydrazines are typical suicide inhibitors of the enzyme. The former act by forming a flavocyanine with N(5) of the covalently bound flavin component (1). Arylhydrazines are oxidized to the corresponding aryldiazene (2), which then inactivates the enzyme, at least in part, by reacting with C(4a) of the flavin (3). The purpose of the present paper is to show that arylcyclopropylamines are also suicide inhibitors but that their target is an amino acid component of the catalytic site, rather than the flavin.

EXPERIMENTAL PROCEDURES

Monoamine oxidase from beef liver was purified as previously described (4). Its concentration was estimated from the stoichiometric binding of [14C]pargyline (4). DL-trans-Phenylcyclopropylamine hydrochloride (PCPA) was obtained from the Sigma Chemical Co. [2-14C]jpm-trans-Phenylcyclopropylamine sulfate, 0.69 Ci/mol, d-trans-phenylcyclopropylamine, and L-trans-phenylcyclopropylamine were the kind gift of D. J. Wilson and C. Kaiser, Smith, Kline and French laboratories.

Monoamine oxidase activity was determined spectrophotometrically by the method of Tabor et al. (5) using benzylamine as substrate. Spectra were recorded with a Cary 14 instrument, interfaced with a Nova 2/4 computer. Other materials and methods were as in previous work (4).

RESULTS

Kinetics of Inactivation—The studies of McEwen et al. (6) clearly suggested that PCPA is an active site-directed, irreversible inhibitor of monoamine oxidase. One observation in disagreement with this was an earlier report (7) that dialysis against substrate nor gel exclusion on columns equili-

brated with substrate resulted in any recovery of the activity. In fact, the data to be presented establish that PCPA is a typical suicide inhibitor of hepatic monoamine oxidase.

The progress of the inactivation at a low concentration of dl-PCPA is shown in Fig. 1. The reaction consists of two second order reactions. The apparent second order rate constant of inactivation for d-PCPA at 30°C and pH 7.2, derived from pseudo-first order kinetics was 5 X 10^3 M^-1 min^-1 (data not shown). From initial rate kinetics an apparent K_i of 4 μM was obtained. Since only the nonprotonated form of the inhibitor is active and its pKa is 8.2 (6), the actual K_i of d-PCPA may be calculated (6) to be 0.36 μM. For l-PCPA the apparent second order rate constant for the inactivation was 6.5 X 10^3 M^-1 min^-1, the apparent K_i was 330 μM, and the K_i corrected for the proper ionizing form was 30 μM. The fast phase in Fig. 1, therefore, represents the inactivation of mono-

amine oxidase by the d-PCPA and the subsequent slow phase by the l-PCPA. The fact that inactivation did not reach

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completion even in 1 h is due to the fact that the concentration of the enzyme exceeded that of PCPA. At higher ratios of inhibitor, enzyme loss of activity was complete in a few minutes and the reaction assumed pseudo-first order kinetic characteristics.

Absorbance Changes during Inactivation—The inactivation of monoamine oxidase is accompanied by complete bleaching of the absorbance of its flavin component in the visible, whether the experiment is carried out aerobically, as in Fig. 2, or anaerobically. The decline of absorbance at 450 nm is as calculated from the molar absorbance of FAD at this wavelength (10.3 x 10^4 M^-1 cm^-1) and dithionite causes no further bleaching. The difference spectra shown in the inset are not entirely characteristic of flavin reduction, as is obtained on treatment of the enzyme with substrate (4), because in addition to bleaching of the 450 nm band they also show a wide shoulder centered around 390 nm.

As expected of an active site-directed irreversible inhibitor, the extent of inactivation during the rapid phase varies linearly with the amount of inhibitor added and is paralleled by a loss of absorbance at 450 nm (Fig. 3). Complete bleaching of the flavin band at 450 nm and full inactivation are reached at a ratio of 1.1 to 1.2 mol of d-PCPA/mol of enzyme. At this point slightly over 1 mol of radioactive inhibitor is tightly bound to the enzyme, as may be ascertained by using ^14C]-PCPA and removing uncombined inhibitor by centrifugation through columns of Sephadex G-25 (8) (Fig. 4).

Fig. 1. The rate of inactivation of monoamine oxidase by PCPA. The enzyme (0.4 μmol) was incubated with 0.025 μmol PCPA in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer containing 1% (w/v) Triton X-100, pH 7.2 at 30°C. At indicated times aliquots were withdrawn and their enzyme activity was compared to the uninhibited enzyme.

Fig. 2. Spectral changes accompanying the inhibition of monoamine oxidase by PCPA. The enzyme (11 μmol) was incubated with 0.625 μmol PCPA in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer containing 1% (w/v) Triton X-100, pH 7.2 at 30°C for a period of 10 min. Aliquots of 150 μl were withdrawn at that time and unbound inhibitor was separated from the enzyme-inhibitor complex by rapid gel filtration through Sephadex G-25. Protein, total counts, and enzyme activity were determined in aliquots of the eluate. The numbers given on the abscissa are for the d,l mixture. They have to be divided by 2 to obtain the quantity of the rapidly reacting d form.

Fig. 3. Comparison of decrease of absorbance at 450 nm and loss of enzyme activity during the reaction of PCPA with monoamine oxidase. The enzyme (11 μmol) was incubated with increasing concentrations of PCPA at 22°C in a buffer system described in the legend of Fig. 1. Spectra were taken after a 10-min incubation period against a reference cuvette containing everything except the enzyme.

Fig. 4. Stoichiometry of binding of[^14C]PCPA to monoamine oxidase. An aliquot of the enzyme (150 μl, 5.6 μmol) was incubated with increasing concentrations of[^14C]PCPA in a total volume of 180 μl at 30°C for a period of 10 min. Aliquots of 150 μl were withdrawn at that time and unbound inhibitor was separated from the enzyme-inhibitor complex by rapid gel filtration through Sephadex G-25. Protein, total counts, and enzyme activity were determined in aliquots of the eluate. The numbers given on the abscissa are for the d,l mixture. They have to be divided by 2 to obtain the quantity of the rapidly reacting d form.
Suicide Inactivation of Monoamine Oxidase

aerobiosis by pargyline, an acetylenic suicide inhibitor (because the oxidized flavin is required for converting pargyline to the inhibitory form), reduction of the flavin by dithionite seemed to have no effect on the inactivation of the enzyme by PCPA.

This conclusion is contradicted by the following experiment. The enzyme (44 μM) was inactivated with 130 μM [14]C-PCPA for 10 min at room temperature. After exhaustive dialysis, the enzyme-inhibitor complex, containing 19,500 cpm, was precipitated with 6.6% (w/v) perchloric acid, containing 0.027 M 2,4-dinitrophenylhydrazine. The reaction mixture was extracted with ethyl acetate and aliquots were chromatographed on silica gel thin layer plates in benzene:methanol (8:2, v/v). The sample derived from the inhibited enzyme gave a single, yellow spot, clearly a 2,4-dinitrophenylhydrazone, which contained 78% of the calculated amount of radioactivity. The same R value was given by a compound prepared by diazotization of PCPA, hydrolysis, controlled oxidation with chromium oxide, and treatment with 2,4-dinitrophenylhydrazine, which is probably 2-phenylcyclopropanone.

It is clear from this experiment that the radioactive inhibitor liberated from the inactivated enzyme reacts with 2,4-dinitrophenylhydrazine and is, therefore, a carbonyl. Thus, dehydrogenation of PCPA by the enzyme must be an integral part of the inactivation process. We suspect that contrary results of Helleman and Erwin (7) were due to the rapid oxidation of PCPA to the imine during the assay, which was conducted aerobically.

Is PCPA a Flavin-directed Inhibitor?—It has been proposed (7) that arylcyclopropanamines combine with monoamine oxidase at the same site as arylhydrazines and acetylenic amines. Subsequent studies (1, 3) demonstrated that both of these two types of suicide inhibitor form covalent adducts with the flavin, the former at C(4a), the latter at N(5). Adduct formation at one of these positions would, of course, explain why the flavin remains bleached in the inactivated enzyme even in the presence of air. Nevertheless, as shown below, the flavin is not the combining site of the inhibitor in the case of PCPA.

The first bit of evidence supporting this conclusion came from the observation that acid, alkali, or heat denaturation liberates from the enzyme the radioactive product formed.

### Table 1

**Release of inhibitor by denaturing agents as function of time**

The enzyme (50 μM) was incubated for 10 min with 200 μM [14]C-PCPA at 30°C in the buffer system described in the legend of Fig. 1. After dialysis, aliquots were mixed at 30°C with 10 M urea or 3% (w/v) sodium dodecyl sulfate in the same buffer to achieve a final concentration of 8 M urea and 1% (w/v) sodium dodecyl sulfate, respectively.

A third sample of the enzyme-inhibitor complex was deaerated and mixed with 10 M urea in buffer under argon at room temperature. At indicated times 15 μl were withdrawn and subjected to rapid gel filtration through Sephadex G-25 equilibrated with 8 M urea and 1% sodium dodecyl sulfate in buffer, respectively. The ratios of the moles of inhibitor remaining bound/mol of enzyme were calculated from the protein concentration and [14]C content in the eluates.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inhibitor remaining bound mol/mol enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.95</td>
</tr>
<tr>
<td>3</td>
<td>0.82</td>
</tr>
<tr>
<td>5</td>
<td>0.75</td>
</tr>
<tr>
<td>10</td>
<td>0.64</td>
</tr>
<tr>
<td>14</td>
<td>0.60</td>
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<tr>
<td>23</td>
<td>0.53</td>
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<tr>
<td>34</td>
<td>0.45</td>
</tr>
<tr>
<td>60</td>
<td>0.41</td>
</tr>
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![Fig. 5. Change of absorbance at 450 nm of monoamine oxidase inhibited by PCPA in the presence of 8 M urea as function of time. The enzyme (50 μM) was incubated with 200 μM PCPA aerobically (a) or anaerobically (b) in the buffer described in the legend of Fig. 1. After dialysis, aliquots of these solutions were mixed with 10 M urea in the same buffer to achieve a final concentration of 8 M urea. Change of absorbance at 450 nm was monitored with a Gilford spectrophotometer at 30°C. In the anaerobic sample air was admitted at the point indicated by the arrow.](http://www.jbc.org/)

![Fig. 6. Chromatography of a trypsin-chymotrypsin digest of monoamine oxidase, previously inhibited with [14]C-PCPA, on Sephadex G-25. The preparation of the sample and chromatography on Sephadex G-25 (0.9 × 82 cm column) in 0.05 M phosphate buffer, pH 7.2, were conducted anaerobically. Fractions of 0.6 ml were collected and their radioactivity and flavin content were determined, the latter after performic acid oxidation.](http://www.jbc.org/)
complete reoxidation of the flavin occurs, even though, at the
time indicated by the arrow in Fig. 5, over 40% of the radio-
active inhibitor was still attached to the enzyme (cf. Fig. 5
and Table I). Hence, on slow denaturation at neutral pH,
conditions may be found when a substantial part of the
inhibitor is still protein-bound but the flavin is nevertheless
fully reoxidized. This is, of course, incompatible with the
suggestion that the inhibitor is bound either at N(5) or at
C(4a) of the flavin.

Further evidence that some component of the enzyme other
than the flavin is the combining site of the inhibitor came
from the experiment of Fig. 6. A sample of monoamine oxidase
inactivated with [14C]PCPA, from which uncombined inhibi-
tor had been removed, was digested with trypsin-chymotryp-
sin at neutral pH and chromatographed on a column of
Sephadex G-25. The entire experiment was conducted under
conditions where C(4a) and N(5) adducts are stable (anaero-
biosis, neutral pH, absence of light). Assays of each fraction
for cysteinyl flavin (4) and for radioactivity demonstrated a
clear separation of the flavin peptide from the labeled
inhibitor. Moreover, upon exposure to air the flavin peptide fraction
was rapidly reoxidized and showed a normal absorption spect-
trum and fluorescence characteristics. This experiment con-
firms that the combining site of the inhibitor is not the flavin
but some amino acid component of the catalytic site. The
probable identity of this site is analyzed under “Discussion.”

**DISCUSSION**

At the outset of this investigation, on the basis of kinetic
studies (6, 7), it appeared likely that arylcyclopropylamines,
as exemplified by PCPA, are suicide inhibitors of monoamine oxidase,
as has been demonstrated for the other two classes of
potent irreversible inhibitors of the enzyme, arylhydrazines
(3), and acetylenic amines (1). Two observations (7) seemed
to contraindicate this hypothesis. One was that dialysis against
substrates causes major reactivation, the other that the oxida-
tion of the enzyme is not required for the development of the
inactivation, implying that oxidation of PCPA by the
enzyme is not required for it to react. As we demonstrated
in our hands no reactivation occurred under any condition
tested. Further, the oxidation of PCPA to 2-phenylcyclopro-
panone by the enzyme has now been experimentally demon-
strated. Thus, arylcyclopropylamines do fulfill all the normal
criteria of suicide inhibition.

Since the initial step in the development of the inactivation
is clearly oxidation by the flavin, it is not surprising that the
450 nm absorption band of the flavin is bleached during this
event. The fact that the flavin remains bleached in the inac-
tivated enzyme in the presence of oxygen, in turn, implies
either that the oxidized inhibitor combines at C(4a) or at N(5)
in a covalent linkage or that the reoxidation of the flavin
moiety in the inactivated enzyme is somehow prevented.
Several observations in this paper rule out the first possibility.
First, denaturation under conditions which are not known to
cleave alkyl- or aryl-substituents from C(4a) or N(5) result in
dissociation of the labeled inhibitor and reoxidation of the
flavin. Second, in proteolytic digests of the inactivated enzyme
the oxidized inhibitor and the flavin appear in different frac-
tions. Third, under relatively mild conditions of denaturation
the flavin exists much faster and more exten-

tively than the release of the labeled inhibitor. We conclude,
therefore, that (a) the inhibitor is attached to an amino acid
component of the catalytic site, not to the flavin, and (b) this
attachment somehow prevents reoxidation of the protein-
bound flavin by oxygen.

As to the identity of the amino acid residue which is the
target of the oxidized inhibitor, Schiff’s base formation with

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**Fig. 7. Possible structures of the adduct of oxidized PCPA and
monoamine oxidase.**

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a primary —NH₂ group may be ruled out since treatment
with NaBH₄, failed to prevent the release of the labeled
inhibitor from the enzyme on drastic denaturation. A more
likely possibility is the —SH group(s) known to be present at
the substrate site since 1945 (10). The nature and function of the

cysteine residues of the enzyme and their relation to
catalytic activity have been extensively studied in several
laboratories in recent years (11-14) and the presence of —SH

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The unusual difference spectrum of the inactivated enzyme
in the 360 to 420 nm region (Fig. 2) may also be due to a perturbation of
the flavin spectrum by an interaction of its benzene nucleus
with that of the inhibitor.

It may be noted that, following the completion of this study,
a paper by Abeles (17) appeared, which postulates, by analogy
Suicide Inactivation of Monoamine Oxidase

with studies on aldehyde dehydrogenase, the structure shown
in Fig. 7 for PCPA-inactivated monoamine oxidase. In con-
trast to the behavior of PCPA, alkylarylcyclopropylamines
were reported by Silverman and Hoffman (18) to form labile
adducts with monoamine oxidase, which have been suggested
to involve N(5) of the flavin.

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